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VERIFICATION OF A TRANSLATION

I, Shoji Sawai, hereby declare that:

My name and post office address are as stated below; that I am knowledgeable in the English language and in the Japanese language, that I translated Japanese patent application no 2002-89624, and that I believe the attached English translation of Japanese patent application no 2002-89624 is a true and complete translation.

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of any application or any patent issuing thereon.

Date: April 30, 2008

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[NAME OF THE DOCUMENT] SPECIFICATION

[NAME OF THE INVENTION] METHOD FOR INDUCING PROLIFERATION OF NEURAL STEM CELLS

[CLAIMS]

[Claim 1] A method for inducing proliferation of a neural stem cell, comprising contacting the neural stem cell with at least one of a dendritic cell, a blood cell or the culture supernatant of the cell, or granulocyte-macrophage colony stimulating factor (GM-CSF).

[Claim 2] The method for inducing proliferation of a neural stem cell of claim 1, comprising contacting the neural stem cell with at least one of a dendritic cell, a blood cell, and granulocyte-macrophage colony stimulating factor (GM-CSF) in a culture medium.

[Claim 3] The method for inducing proliferation of a neural stem cell of claim 2, comprising isolating a mammalian nervous tissue containing the neural stem cell, selectively culturing the neural stem cell in a culture medium containing a growth factor, and then co-culturing the neural stem cell with a dendritic cell and/or a blood cell.

[Claim 4] The method for inducing proliferation of a neural stem cell of claim 2, comprising isolating a nervous tissue containing the neural stem cell, selectively culturing the neural stem cell in a culture medium containing a growth factor, and then culturing the neural stem cell in the culture supernatant of at least one of a dendritic cell and a blood cell.

[Claim 5] The method for inducing proliferation of a neural stem cell of any one of claims 2 to 4, wherein the culture medium containing the growth factor is a culture medium containing EGF and/or FGF.

[Claim 6] The method for inducing proliferation of a neural stem cell of any one of claims 1 to 5, wherein the dendritic cell is an immature dendritic cell subset having the CD11c surface marker on the cell surface or a mature dendritic cell subset derived from the immature dendritic cell subset.

[Claim 7] The method for inducing proliferation of a neural

stem cell of any one of claims 1 to 6, wherein the blood cell is a spleen cell, a T cell, a monocyte, a neutrophil, an eosinophil, or an basophil.

[Claim 8] A set for inducing proliferation of a neural stem cell, comprising at least one of a dendritic cell, a blood cell or the culture supernatant of the cell, or granulocyte-macrophage colony stimulating factor (GM-CSF).

[Claim 9] The set for inducing proliferation of a neural stem cell of claim 8, further comprising a culture medium containing a growth factor.

[Claim 10] The set for inducing proliferation of a neural stem cell of claim 9, wherein the culture medium containing the growth factor is a culture medium containing at least EGF and/or FGF.

[Claim 11] The set for inducing proliferation of a neural stem cell of any one of claims 8 to 10, wherein the dendritic cell is an immature dendritic cell subset having the CD11c surface marker on the cell surface or a mature dendritic cell subset derived from the immature dendritic cell subset.

[Claim 12] The set for inducing proliferation of a neural stem cell of any one of claims 8 to 11, wherein the blood cell is a spleen cell, a T cell, a monocyte, a neutrophil, an eosinophil, or a basophil.

[DETAIL DESCRIPTION OF THE INVENTION]

[0001]

[TECHNICAL FIELD]

The present invention relates to methods for inducing proliferation of neural stem cells, which are undifferentiated neural cells with pluripotency, use of neural stem cells obtained by such proliferation-inducing methods, proliferation-inducing sets of neural stem cells, and use of such proliferation-inducing sets of neural stem cells.

[0002]

[BACKGROUND ART]

Many spinal cord injuries are traumatic and they are caused by traffic accidents, sport accidents, industrial

accidents and the like. Non-traumatic ones are attributed to inflammation, bleeding, tumors, spine deformation and the like. The pathologic conditions include crush and pressure lesions of the spinal cord based on bleeding and edema in the spinal substance, causing neuropathy corresponding to the injured site. As main clinical manifestations, paresi or paralysis and anesthesia occur below the injury level. In the case of cervical cord injuries, respiratory paralysis and hyperthermia (or hypothermia) are observed as the characteristic complications. The improvement of the above-mentioned neuropathy, especially the amelioration of dyskinesia is directly linked to prevention of the increasing number of the bedridden elderlies or to enhancement of their quality of life (QOL); it is becoming more important with the increasing average life span in recent years.

[0003]

Treatment for the above-mentioned spinal cord injuries include surgical operations for eliminating physical compression and injury and steroid therapy for spine edema in the acute phase after injury (N.Engl J. Med. 322, 1405-1411, 1990, J.Neurosurg 93, 1-7, 2000). It is reported that among steroids, high-dose administration of methylprednisolone is effective in improving neurological symptoms associated with spinal cord injuries (J.Spinal Disord. 5(1), 125-131, 1992). However, excessive administration of steroids is also accompanied by strong systemic side effects and difficult to control. In addition, in the case of the spinal cord injuries accompanied by an infection, such administration causes a problem of reducing the function of defending against infection. Furthermore, currently, the validity of high-dose steroid therapy itself has become controversial. As described above, to date, there have been no effective therapeutic agents for spinal cord injuries; the development of a new therapeutic agent is desired.

[0004]

There are other reports on a therapeutic method for

spinal cord injuries: the method for transplanting a therapeutically effective dose of astrocytes pretreated by an inflammation-related cytokine *in vitro* to the injured site in the central nervous system (CNS)(National Publication of International Patent Application No. 2000-503983); and the method for promoting neurocladism in the mammalian CNS by administering monocular phagocytes (monocytes, macrophages, etc.) of the same kind to the injured or disease site, or to the CNS in the vicinity of such sites (J. Mol. Med.77, 713-717, 1999, and N.Neurosci. 19 (5) 1708-16, 1999, Neurosurgery 44 (5), 1041-5, 1999, Trends. Neurosci 22 (7), 295-9, 1999, National Publication of International Patent Application No. 1999-13370, etc.). In addition, there is another report that the recovery of movement maintenance after a spinal cord injury was promoted by vaccination with spinal cord homogenate or administration of T cells specific to the myelin basic protein, which is a myelin sheath protein, although a clear mechanism of the action is not known (Neuron 24, 639-647, 1999, Lancet 354,286-287, 2000).

[0005]

Recently in Europe and America, a clinical trial has been carried out in which embryonic brain cells were transplanted into patients with Parkinson's disease, in which dopaminergic neurons in the mesencephalic substantia nigra are degenerated and lost (Piccini P., et al., Nat Neurosci., 2, 1999, Freed C.R., et al., N.Engl.J.Med., 344, 2001). It was revealed that this treatment method improves the movement ability of patients under the age of 60. It is thought that to perform this transplantation therapy on one patient with Parkinson's disease, as many as 5 to 10 aborted embryos are required.

[0006]

On the other hand, a selective culture method of neural stem cells called the neurosphere method was developed by the group of Weiss et al. in 1992, whereby a turning-point was marked in the history of studies of neural stem cells (Reynolds B.A, et al.J.Neurosci., 12, 1992). In this method, cell colonies containing neural stem cells are cultured in a serum-free liquid

medium containing some growth factor(s). Here, only neural stem cells proliferate and suspend as cell aggregates (neurospheres). Furthermore, when the neurospheres that have generated are disaggregated into individual cells and cultured in the aforementioned serum-free liquid medium again, neurospheres are formed similarly. When these neurospheres are cultured in the aforementioned serum-free liquid medium without the growth factor(s), cell differentiation is induced so that three kinds of cells, neurons, astrocytes, and oligodendrocytes are formed.

[0007]

Meanwhile, dendritic cells (DCs) are a population of cells with the arborescent form, derived from hematopoietic stem cells, and are distributed widely in the living body. Immature dendritic cells recognize and incorporate foreign substances, including viruses and bacteria that have invaded individual tissues, digest and degrade them to generate peptides in the process of moving to the T cell region of the lymphoid organs, and bind the peptides to MHC molecules and present them on the cell surface, thereby bearing a role of antigen-presenting cells that activate antigen-specific T cells and induce immunological responses (Ann.Rev.Immunol.9, 271-296, 1991, J.Exp.Med.185, 2133-2141, 1997).

[0008]

Although dendritic cells are distributed widely, their density in individual tissues was not high; it was difficult to prepare dendritic cells in large numbers. However, addition of some differentiation/growth factor(s) to the culture of immature precursors has enabled easy *in vitro* preparation of a multitude of dendritic cells. Based on that, use of dendritic cells as an immunopotentiator has started to be discussed (J. Exp. Med. 183, 7- 11, 1996). For example, in a feeble tumor immunological response, immunological responses are specifically enhanced by pulsing dendritic cells with antigens. Animal experiments have shown that dendritic cells that have presented a tumor-derived protein or antigen peptide induce

specific CD8 + cytotoxic T cells. It is reported that likewise in humans some tumors are reduced or lost by returning a tumor-derived protein or antigen peptide to the living body together with dendritic cells.

[0009]

[PROBLEMS TO BE SOLVED BY THE INVENTION]

Neural stem cells are undifferentiated neural cells having capability, i.e., pluripotency, to differentiate into three kinds of cells constituting the central nervous system, called neurons, astrocytes, and oligodendrocytes, together with a self-replication ability to divide and proliferate (Temple S., Nature, 414, 2001). In recent years, it has been found that neural stem cells are present even in the adult brain with extremely low regenerative capacity. Further, isolation and preparation of human neural stem cells have become possible. Thus neural stem cells have now become the focus of special attention in the current regenerative medicine research.

[0010]

Recently, a method has been developed for inducing differentiation of stem cells that have proliferated *in vitro* into dopaminergic neurons. If transplantation of cells induced by this method as donor cells becomes possible, that is expected to be a therapeutic method for Parkinson's disease far more superior to the current method requiring a large number of aborted embryos. In addition, it is expected that such therapy as transplantion of cells prepared in large quantities by getting stem cell biology into full use will be performed on various neurological diseases from now on. An object of the present invention is to provide methods, which are the most important for such transplantation therapy, for efficiently inducing proliferation of neural stem cells *in vitro* and in other manners, and others.

[0011]

[METHOD TO SOLVE THE PROBLEMS]

It is considered that in the adult spinal cord injuries, while endogenous neural stem cells are present within the spinal

cord, de novo neurogenesis is suppressed and proliferation of astrocytes alone occurs. Since simple introduction of neural stem cells into an injured site will result in glial formation, without neuronal formation, such treatment is unlikely to lead to improvement of the pathologic condition. Therefore, in addition to neural stem cell transplantation, arrangement of the microenvironment for producing neurons is essential. On the other hand, an antigen-specific immunological reaction mainly involving T cells is present as one of the host defense mechanisms. The central nervous system is in the unique environment completely isolated from the immune system because of the presence of the blood brain barrier, extremely low expression of MHC antigens, lack of the lymphatic tissue, and so forth. Thus, based on the assumption that the immune system eliminates and restores the diseased tissue, the present inventors attempted introduction of the immune system to the injured nervous tissue. Specifically, the inventors found that proliferation of endogenous neural stem cells are induced by administering, to injured nervous tissue, dendritic cells, the most important cells for regulating the immune system, and/or granulocyte-macrophage colony stimulating factor (GM-CSF), a cytokine important for induction and proliferation of dendritic cells. The inventors also found that *in vitro* proliferation of neural stem cell is induced by co-culture with dendritic cells. The present invention has thus been accomplished.

[0012]

The present invention relates to a method for inducing proliferation of a neural stem cell, including contacting the neural stem cell with at least one of a dendritic cell, a blood cell, the culture supernatant of the cell, or granulocyte-macrophage colony stimulating factor (GM-CSF) (claim 1); the method for inducing proliferation of a neural stem cell of claim 1, including contacting the neural stem cell with at least one of a dendritic cell, a blood cell, and granulocyte-macrophage colony stimulating factor (GM-CSF)in a

culture medium (claim 2); the method for inducing proliferation of a neural stem cell of claim 2, including isolating a mammalian nervous tissue containing the neural stem selectively culturing the neural stem cell in a culture medium containing a growth factor, and then co-culturing the neural stem cell with a dendritic cell and/or a blood cell (claim 3); the method for inducing proliferation of a neural stem cell of claim 2, including isolating a mammalian nervous tissue containing the neural stem cell, selectively culturing the neural stem cell in a culture medium containing a growth factor, and then culturing the neural stem cell in the culture supernatant of a dendritic cell and/or a blood cell (claim 4); the method for inducing proliferation of a neural stem cell of any one of claims 2 to 4, in which the culture medium containing the growth factor is a culture medium containing at least EGF and/or FGF (claim 5); the method for inducing proliferation of a neural stem cell of any one of claims 1 to 5, in which the dendritic cell are an immature dendritic cell subset having the CD11c surface marker on the cell surface or a mature dendritic cell subset derived from the immature dendritic cell subset (claim 6); and the method for inducing proliferation of a neural stem cell of any one of claims 1 to 6, in which the blood cell is a spleen cell, a T cell, a monocyte, a neutrophil, an eosinophil, or a basophil (claim 7).

[0013]

The present invention also relates to a set for inducing proliferation of a neural stem cell, including at least one of a dendritic cell, a blood cell or the culture supernatant of the cell, or the granulocyte-macrophage colony stimulating factor (GM-CSF) (claim 8); the set for inducing proliferation of a neural stem cell of claim 8, further including a culture medium containing a growth factor (claim9); and the set for inducing proliferation of a neural stem cell of claim 9, in which the culture medium containing the growth factor is a culture medium containing at least EGF and/or FGF (claim 10); the set for inducing proliferation of a neural stem cell of any one of

claims 8 to 10, in which the dendritic cell is an immature dendritic cell subset having the CD11c surface marker on the cell surface or a mature dendritic cell subset derived from the immature dendritic cell subset (claim 11), the set for inducing proliferation of a neural stem cell of any one of claims 8 to 11, in which the blood cell is a spleen cell, a T cell, a monocyte, a neutrophil, an eosinophil, or a basophil (claim 12).

[0014]

[EMBODIMENTS]

The method for inducing proliferation of neural stem cells according to the present invention is not particularly limited, whether *in vitro, in vivo,* or *ex vivo*, as long as it is a method in which neural stem cells are contacted with at least one of dendritic cells, blood cells or culture supernatant of these cells, or granulocyte-macrophage colony stimulating factor (GM-CSF). In consideration of transplantation therapy requiring neural stem cells prepared in large quantities, however, a preferred method is to induce proliferation of neural stem cells, by contacting neural stem cells with at least one selected from a group consisting of dendritic cells, blood cells, and granulocyte-macrophage colony stimulating factor (GM-CSF) in a culture medium such as DMEM/F12 medium. Illustratively, a method for inducing proliferation of neural stem cells is to isolate and harvest mammalian nervous tissues (e.g., the putamen-corpus striatum of a fetus) containing the neural stem cells with the pluripotency to differentiate into three kinds of cells constituting the central nervous system, called neuron, astrocytes, and oligodendrocytes, together with a self-replication ability to divide and proliferate, selectively culture the neural stem cells in a culture medium containing growth factors, and subject the neural stem cells to a treatment for increasing the purity of the neural stem cells as necessary, followed by contacting the neural stem cells with dendritic cells and/or blood cells by co-culturing these cells in a culture medium. Another method for inducing proliferation of neural stem cells, for example, is to contact neural stem

cells with GM-CSF by culturing them in the presence of GM-CSF, regardless of the presence or absence of co-culture with the above-mentioned dendritic cells and/or blood cells. Yet another preferred method for inducing proliferation of neural stem cells is illustratively to isolate nervous tissues containing the neural stem cells, selectively culture the neural stem cells in a culture medium containing growth factors like the above described, and then contact the neural stem cells by culturing them in the culture supernatant of dendritic cells and/or the culture supernatant of blood cells.

[0015]

As the above-mentioned growth factors, epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor α (TGFα), amphiregulin, betacellulin (BTC), epiregulin (ER), heparin-binding EGF-like growth factor (HB-EGF), schwannoma-derived growth factor (SDGF), etc. can be illustratively shown; especially, EGF and FGF are illustratively preferred. Further, to a culture medium containing the above-mentioned growth factor(s), ingredients usually used for cell culture, such as transferrin, insulin, retinoic acid, activin, interleukin, etc. other than such growth factors as EGF and FGF, may be added.

[0016]

Illustratively, the aforementioned dendritic cells are preferably an immature dendritic cell subset having the CD11c surface marker on the cell surface or a mature dendritic cell subset derived from the immature dendritic cell subset. Such dendritic cell subset includes a dendritic cell subset that secrete neurotrophic factor NT-3, which exerts *in vivo* nerve-regeneration effect, and induces proliferation and enhancement of phagocytosis of microglia; an immature dendritic cell subset that express, in addition to NT-3, CNTF (neurotrophic factor), which has the effect of protection from degeneration/cell death on both the motor and sensory nerves of the spinal cord, TGF-β1, which has the inhibitory effect on

release of cytotoxic substances derived from microglia or macrophages, and IL-6, which induces the protection effect on various neurons (cholinergic, catecholaminergic, and dopaminergic neurons); and a mature dendritic cell subset that express EGF whose neuroprotective effect has been confirmed, CNTF, TGF-β1, and IL-6, in addition to NT-3. Alternatively, as the above-described mature dendritic cell subset, a mature dendritic cell subset obtained by culturing an immature dendritic cell subset *in vitro* in the presence of stimulants for maturing immature dendritic cells, such as LPS, IL-1, TNF-α, and CD 40L, may be used.

[0017]

An immature dendritic cell subset having the CD11c surface marker on the cell surface can be obtained by isolating the dendritic cell subset by, for example, using a method of subjecting peripheral blood etc. to the pretreatment of density centrifugal separation treatment, etc., followed by sorting by FACS using a monoclonal antibody against a dendritic cell surface antigen or a separation method using a magnetic bead-coupled monoclonal antibody against dendritic cell surface antigen or the like, and then selecting the CD11c-positive dendritic cell subset out of the dendritic cell subset. Moreover, dendritic cells to be contacted with neural stem cells are preferably of the same kind as these neural stem cells. The preferred number of dendritic cells to be contacted with neural stem cells is 10^3 or more of that of the neural stem cells in terms of the ratio of the number of cells because of marked induction of neural stem cell proliferation.

[0018]

The aforementioned blood cells are illustratively spleen cells, T cells, monocytes, neutrophils, eosinophils, and basophils. Among these, T cells, especially CD8-positive T cells, and spleen cells are illustratively preferred.

[0019]

Next, the set for inducing proliferation of neural stem cells according to the present invention is not particularly

limited as long as it includes at least one kind of dendritic cells, blood cells or a culture supernatant of these cells, or GM-CSF. However, preferred are the sets that further include a culture medium containing various ingredients such as growth factors including EGF and FGF. Further, the aforementioned dendritic cells are preferably an immature dendritic cell subset having the CD11c surface marker on the cell surface or a mature dendritic cell subset derived from the immature dendritic cell subset. The blood cells are preferably spleen cells, T cells, monocytes, neutrophils, eosinophils, or basophils.

[0020]

Treatment of illnesses, such as degenerative diseases, such as Parkinson's disease, Alzheimer's disease, and Huntington disease, traumatic and neurotoxic injuries to the central nervous system, and cerebral infarction resulting from the inhibition of blood flow or oxygen supply to the nervous system, etc. become possible by performing the method for inducing proliferation of neural stem cells according to the present invention *in vitro* or *ex vivo* and using the resulting neural stem cells, or performing the method for inducing proliferation of neural stem cells according to the present invention *in vivo*. Therefore, the sets for inducing proliferation thereof obtained by the present invention are useful as therapeutic agents for the above-mentioned nerve injuries or nerve function insufficiency.

[0021]

When using the set for inducing proliferation of neural stem cells as a therapeutic agent for nerve injuries or nerve function insufficiency, various mixing ingredients for preparation, such as a pharmaceutically acceptable common carrier, a binder, a stabilizer, an excipient, a diluent, a pH buffer, a disintegrator, a solubilizer, a solubilizing agent, and a tension agent, may be added to the aforementioned cancer therapeutic agent according to the present invention. Further, such a therapeutic agent may be administered orally or

parenterally. That is, it may be administered orally in commonly used administration forms, for example, such as powder, granules, capsule, syrup, and suspension, or parenterally and locally in the form of an injection in dose forms such as a solution, an emulsion, and a suspension. The agent may also be administered in a spray form into the nostrils.

[0022]

[EXAMPLES]

The present invention will be more specifically described by giving examples hereinbelow. However, the scope of the present invention is not limited to these examples.

EXAMPLE 1 (ISOLATION OF DENDRITIC CELLS)

Immature dendritic cells were obtained by isolating a CD11c-positive subset from spleens of 6-week-old BALB/c or C57BL/6 female mice by the immunomagnetic bead method. Specifically, the spleens were first homogenated with 100 U/ml collagenase (Worthington Biochemical Corporation). Subsequently, the pellicle portion that was hard to separate was further incubated with 100 U/ ml collagenase for 20 min in 5% CO_2 at 37°C, and cells were separated. The cells obtained were suspended in 35% BSA solution, further overlaid with RPMI1640 + 10% fetus serum in a centrifuge tube, and then subjected to centrifugation at 3000 rpm for 30 min at 4°C. The cells at the interface layer between the 35% BSA solution and the RPMI1640 + 10% fetus serum solution were recovered. Next, by reacting for 15 min at 4°C magnetic bead-coupled monoclonal antibody (2×10^8 beads, Miltenyi Biotech) against CD11c antigen with the recovered cells and magnetically separating the cells bound to the beads, the fraction in which the immature dendritic cell subset was concentrated was obtained.

[0023]

EXAMPLE 2 (INDUCTION OF PROLIFERATION OF ENDOGENOUS NEURAL STEM CELLS/ PRECURSORS BY TRANSPLANTATION OF DENDRITIC CELLS)

A laminectomy of the eighth thoracic vertebra was performed on six-week old BALB/c or C57BL/6 mice under ether anesthesia and by hemisecting the spinal cord on the left side

with a scalpel, spinal cord-injured model mice were prepared. Immediately after injury, RPMI1640 medium with or without dendritic cells (1 x 10^5 /mouse) obtained by sorting a CD11c (+) subset using the immunomagnetic bead method was transplanted into the injured site.

[0024]

To examine the reactivity of endogenous neural stem cells/precursors by transplantation of dendritic cells, using Musashi-1 antibody, which recognizes the cells, immunohistochemical staining was performed to investigate time-course changes in the number of positive cells. First, dendritic cell-transplanted mice on day 2, 4, and 7 after injury were subjected to perfusion fixation with 2% paraformaldehyde through the heart and frozen sections were prepared (n=3/group). Next, immunohistological staining was performed using anti-mouse Musashi-1 antibody as the primary antibody. Musashi-1 is an RNA-binding protein with a molecular weight of about 38 kDa, identified by Okano et al. in 1994 (Neuron, 1994). It has been reported that Musashi-1 is strongly expressed in neural stem cells/precursors in the analysis using monoclonal antibody against mouse Musashi-1 (Dev. Biol. 1996, Neurosci. 1997, Dev. Neurosci. 2000). The measurement area was classified into two regions: the proximal and distal injured regions (cranial and caudal) as the regions from dorsal to ventral in each of the most distal region of the gelfoam (degenerative collagen), used when transplanting cells, and the point 1 mm away therefrom. (See FIG. 1).

[0025]

FIG.2 shows staining images of representative sections cranially from the proximal injured region. On day 2 after injury, no difference was observed in both groups, but on and after day 4 after injury a large number of Musashi-1-positive cells were observed in the dendritic cell-transplanted group. In contrast, almost no such changes were found in the control group.

[0026]

Next, Musashi-1-positive cells were quantitatively analyzed using an image analyzing device (Flovel Co., Ltd.). FIG.3 shows time-course changes in the number of Musashi-1-positive cells according to the area. On and after day 4 after injury, a significant increase in the number of Musashi-1 positive cells due to transplantation of dendritic cells was observed both in the proximal and distal injured regions, as compared with the control. Especially, in the proximal injured region, a remarkable increase in the number of Musashi-1-positive cells was observed between day 2 and day 4 after injury in the dendritic cell-transplanted group.

In conclusion, it was revealed that transplantation of dendritic-cells to an injured site induces proliferation of endogenous neural stem cells/precursors.

[0027]

EXAMPLE 3 (INDUCTION OF PROLIFERATION OF ENDOGENOUS NEURAL STEM CELLS / PRECURSORS BY ADMINISTRATION OF GM-CSF)

To analyze the reactivity to neural stem cells/precursors in the central nervous system due to administration of GM-CSF, which is a cytokine important for induction and proliferation of dendritic cells, using Musashi-1 antibody, which recognizes these cells, immunohistological staining was performed for investigation of time-course changes in the number of positive cells. Spinal cord-injured model mice were prepared using six-week-old BALB/c female mice. Immediately after injury, 5 μl of normal saline with or without GM-CFS (250 pg/mouse, Genzyme Corporation) was administered to the injured site of the spinal cord (n=3/group). On days 2, 4, and 7 after injury, the mice were subjected to perfusion fixation with 2% paraformaldehyde through the heart and frozen sections were prepared. Next, immunohistological staining using anti-Musashi-1 antibody as the primary antibody was performed. As for the measurement area, the regions 0.5 mm dorsally and ventrally away from the most distal region of the gelfoam used in the cell transplantation, were quantitatively analyzed using an image analyzing device (Flovel Co., Ltd.)

(FIG.4). Time-course changes in the number of Musashi-1 positive cells are shown in FIG.5. In the GM-CSF-administrated group, a large number of Musashi-1-positive cells were observed as early as day 2 after injury, as compared with the control, and a significant increase in the number of cells was observed on day 7. In conclusion, it was found that administration of GM-CSF to an injured site induces proliferation of endogenous neural stem cells/precursors within nervous tissue.

[0028]

EXAMPLE 4 (*IN VITRO* INDUCTION OF NEURAL STEM CELL PROLIFERATION BY CO-CULTURE WITH DENDRITIC CELLS)

Since it was found that transplantation of dendritic cells into an injured site induces proliferation of endogenous neural stem cells/precursors, it was analyzed whether or not dendritic cells can cause neural stem cell to proliferate *in vitro* as well. Induction of proliferation of neural stem cells was attempted by two-step isolation culture of neural stem cells. In the first step, the putamen-corpus striatum sites of C57BL/6 fetuses (on day 14 of gestation) were isolated and neural stem cells were cultured selectively by culturing the cells for 5 to 7 days at a cell density of 1×10^5 cells/ml in a liquid medium composed of DMEM/F12 medium supplemented with 20 ng/ml EGF (PeproTech, Inc.), 20 ng/ml FGF-2 (R&D Systems), 100 μg/ml transferrin (Sigma Chemical Corporation), 25 μg /ml insulin (Sigma Chemical Corporation), 20 nM progesterone (Sigma Chemical Corporation), 30 nM sodium selenate (Sigma Chemical Corporation), and 60 μM putrescine (Sigma Chemical Corporation).

[0029]

Further, to increase the purity of the neural stem cells obtained, PI staining-negative cells with a diameter of 10 μm or more were isolated using a cell sorter and plated at a density of 100 cells/well, and thus co-culture with dendritic cells was started. The cell sorter used was a Becton Dickenson FACS Vantege SE and for analysis Clone cyte plus was used. For the dendritic cells to be used for co-culture with neural stem cells,

the CD11c-positive subset was isolated from the spleens of mature C57BL/6 female mice and prepared in the above-described liquid medium at a cell density of 1 x 10^3 to 10^5 cell/ml, and 100 μl of the cell suspension was added to each well of a 96-well low-adhesion culture plate. A group to which cells were not added (the fundamental condition for culture of neurospheres) and another group to which NT-3 (1 to 10 ng/ml), the most important neurotrophic factor secreted by dendritic cells, was added, were included as controls. The neural stem cells were thus cultured.

[0030]

Since proliferated neural stem cells form cell aggregates, called neurosphere with a diameter of 50 μm or more, the number (FIG. 6) and the volume (FIG. 7) of neurospheres were measured and examined under individual conditions eight days after co-culture was started. The results revealed that neural stem cells had markedly proliferated due to co-culture with dendritic cells (DC), as compared with the conventional conditions for culture of neurospheres.

[0031]

EXAMPLE 5 (*IN VITRO* INDUCTION OF NEURAL STEM CELL PROLIFERATION USING DENDRITIC CELL CULTURE SUPERNATANT)

Further it was examined whether or not the substance secreted by dendritic cells can induce proliferation of neural stem cells. In this experiment, the number and volume of neurospheres were analyzed using the culture supernatants of not only dendritic cells but also spleen cells and T cells, which are blood cells. Neural stem cells were isolated using the same procedure as described in Example 4. That is, the putamen-corpus striatum sites of C57B L/6 fetuses (on day 14 of gestation) were isolated and neural stem cells were cultured selectively by culturing for 5 to 7 days at a cell density of 1 x 10^5 cells/ ml in a liquid medium composed of DMEM/F12 medium supplemented with 20 ng/ml EGF (PeproTech, Inc.), 20 ng/ml FGF-2 (R&D Systems), 100 μg/ml transferrin (Sigma Chemical Corporation), 25 μg/ml insulin (Sigma Chemical Corporation),

20 nM progesterone (Sigma Chemical Corporation), 30 nM sodium selenate (Sigma Chemical Corporation), and 60 nM putrescine (Sigma Chemical Corporation). Further, to increase the purity of the neural stem cells obtained, after isolating PI staining-negative cells with a diameter of 10 μm or more using a cell sorter, the cells were plated at a density of 100 cells/ml.

[0032]

Spleen cells were prepared from the spleens of mature C57BL/6 female mice. The CD11c-positive subset and the CD8-positive subset were isolated as dendritic cells and CD8T cells, respectively, and cultured in the aforementioned liquid medium for 24 hours. Subsequently, the culture supernatants were recovered. A group to which cells were not added (the fundamental condition for culture of neurospheres) was included as control and neural stem cells were cultured. Since proliferated neural stem cells form cell aggregates, called neurosphere with a diameter of 50 μm or more, eight days after co-culture was started, the number (FIG. 8) and volume (FIG.9) of neurospheres were measured and examined under individual conditions. The results revealed that neural stem cells had markedly proliferated due to the culture supernatants of not only dendritic cells (DCs) but also spleen cells (SPCs) and CD8-positive T cells (CD8-T), as compared with the conventional conditions under which neurospheres are cultured.

[0033]

[EFFECTS OF THE INVENTION]

According to the present invention, it is possible to efficiently induce proliferation of neural stem cells *in vivo* and/or *in vitro*, which are the most important for transplantation therapy and so forth of nerve injuries or nerve function insufficiency.

[BRIEF DESCRIPTION OF THE DRAWINGS]

[Fig. 1]

This shows the area setting for measurement of the number of Musashi-1-positive cells after transplantation of dendritic cells.

[Fig. 2]

This shows the results of immunostaining using anti-Musashi-1 antibody in the dendritic cell (DC)- and the RPMI1640 (RPMI) -transplanted groups, especially representative time-course tissue sections cranially from proximal injured region.

[Fig. 3]

This shows time-course changes in the number of Musashi-1-positive cells in each of the regions in the dendritic cell- and the RPMI1640-transplanted groups.

[Fig. 4]

This shows the area setting for measurement of the number of Musashi-1 positive cells after administration of GM-CSF.

[Fig. 5]

This shows the time-course changes in the number of Musashi-1 positive cells after administration of GM-CSF.

[Fig. 6]

This shows the numbers of neurospheres formed by co-culture with dendritic cells, each indicating an average value of four wells analyzed.

[Fig. 7]

This shows the volumes of the neurospheres formed by co-culture with dendritic cells, each indicating an average value of four wells analyzed.

[Fig. 8]

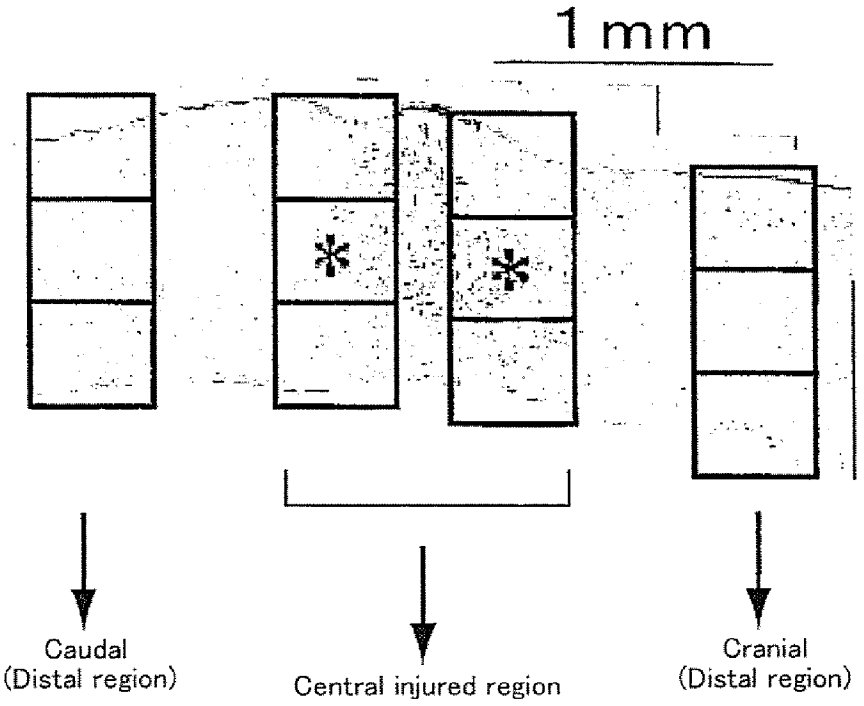
This shows the numbers of neurospheres formed by the culture using the culture supernatants of dendritic cells, spleen cells, and T cells, each indicating an average value of four wells analyzed.

[Fig. 9]

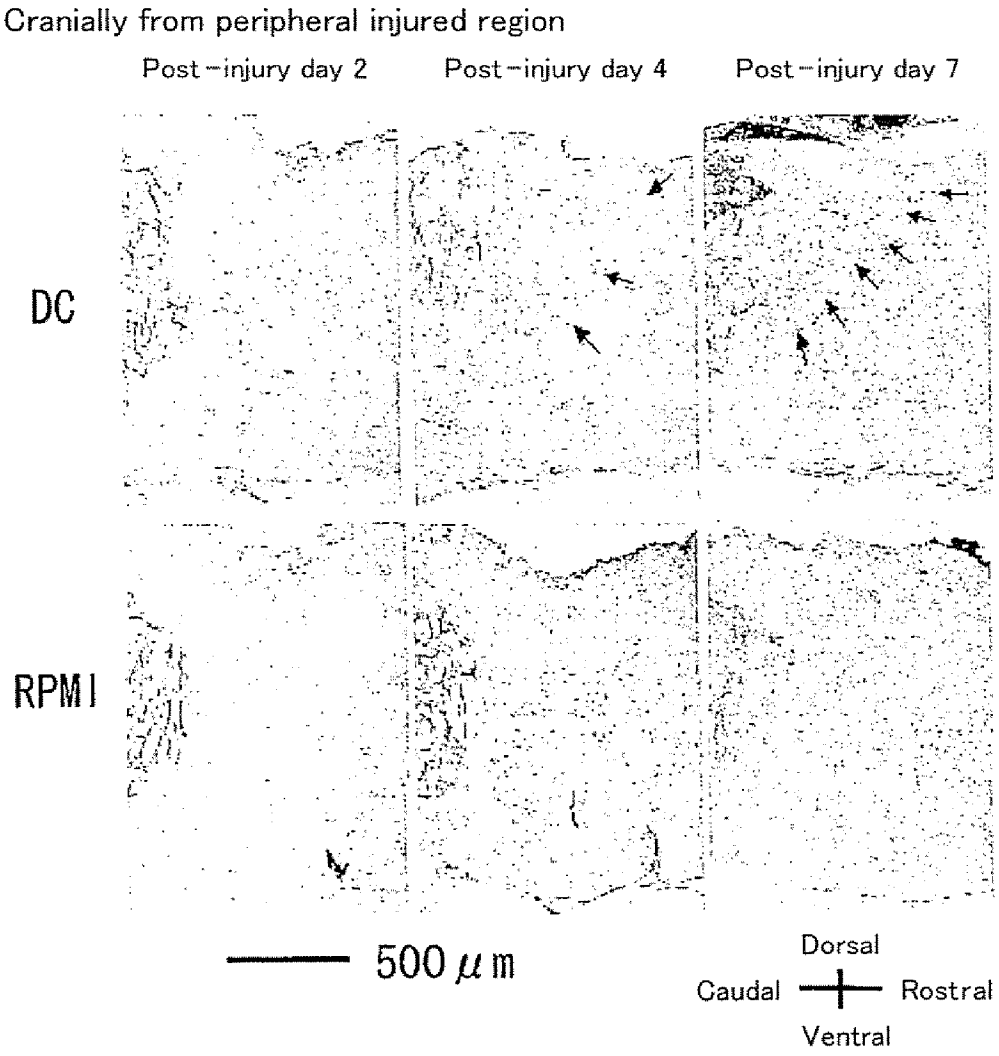
This shows the volumes of neurospheres formed by the culture using the culture supernatants of dendritic cells, spleen cells, and T cells, each representing an average value of neurospheres formed.

[Name of the document] Figures

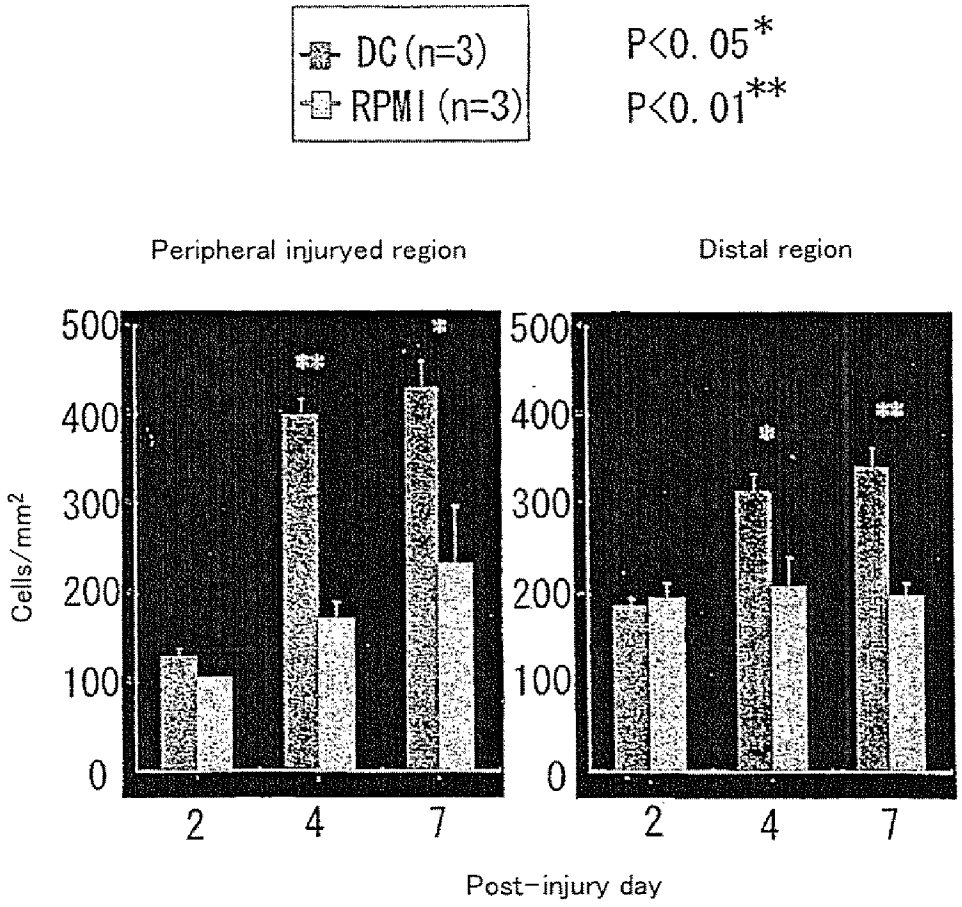
[Fig.1]



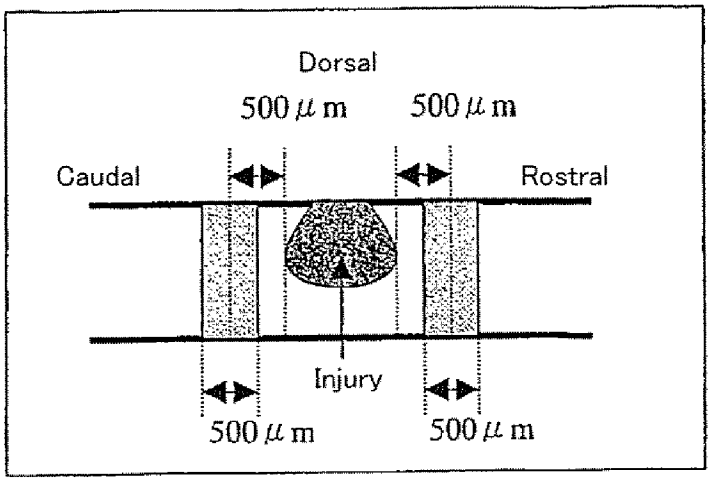
[Fig.2]


Cranially from peripheral injured region
Post-injury day 2
Post-injury day 4
Post-injury day 7
DC
RPMI
500 μm
Dorsal
Caudal
Rostral
Ventral

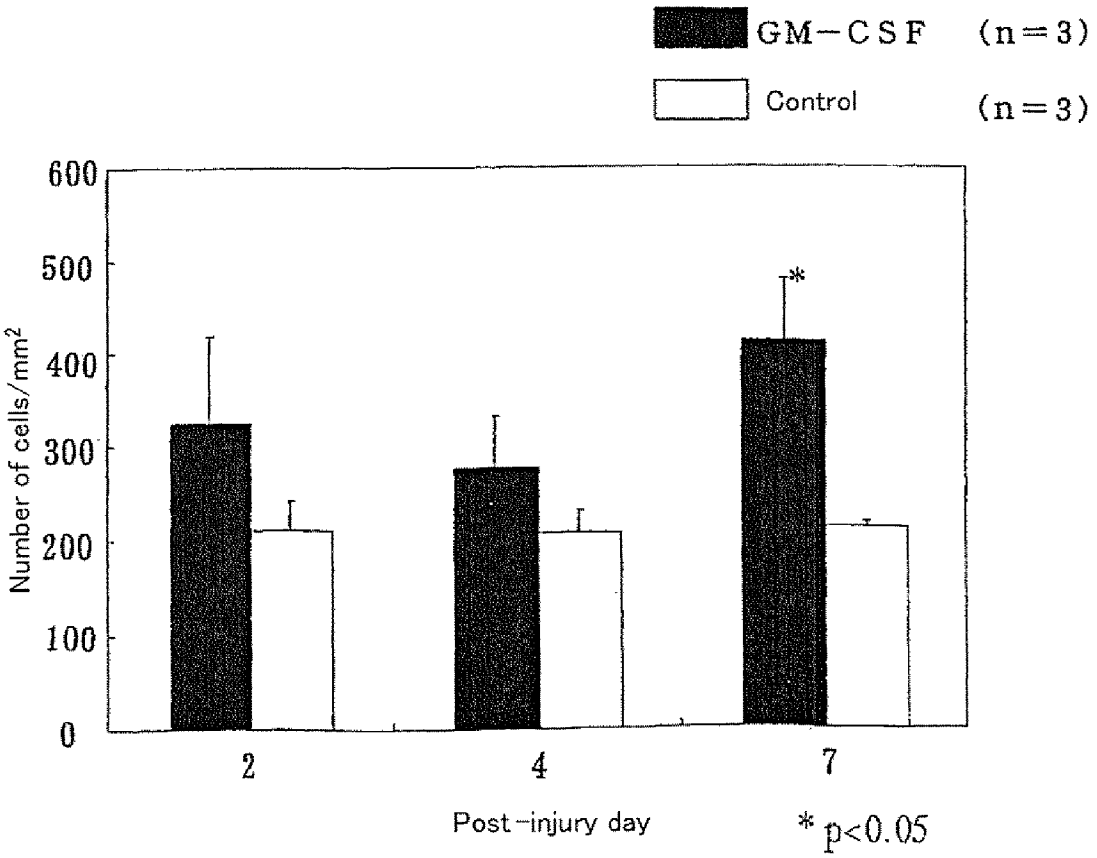
[Fig.3]


DC (n=3)
RPMI (n=3)
P<0.05*
P<0.01**
Peripheral injuryed region
Distal region
Cells/mm2
500
400
300
200
100
0
2
4
7
Post-injury day

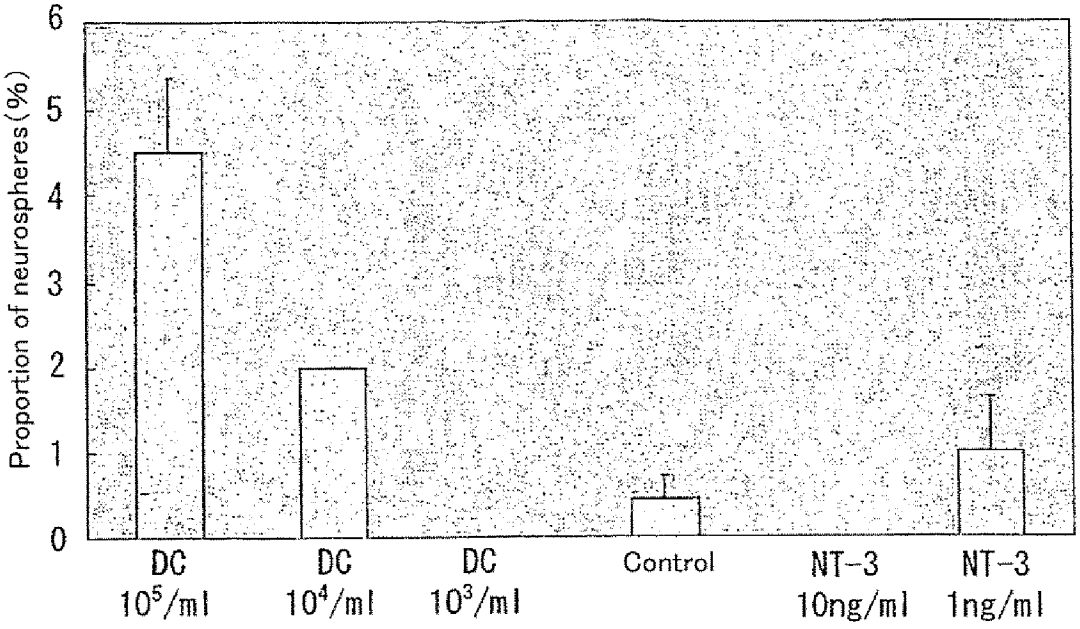
[Fig.4]


Dorsal
500 μm
500 μm
Caudal
Rostral
Injury
500 μm
500 μm

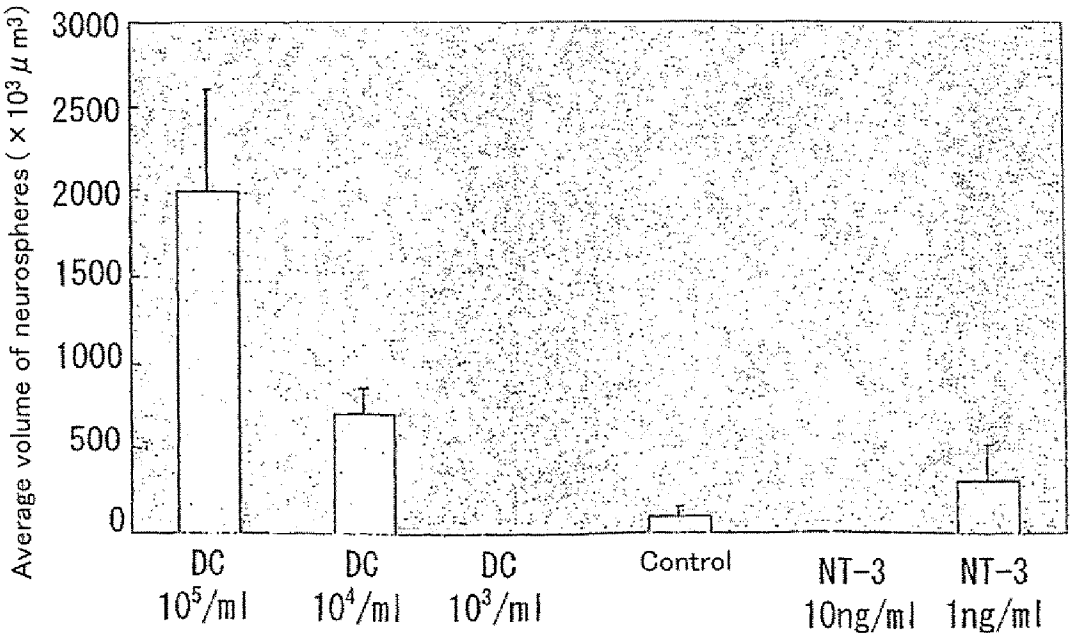
[Fig.5]


GM−CSF (n=3)
Control (n=3)
600
500
400
300
200
100
0
Number of cells/mm²
*
2
4
7
Post-injury day
* p<0.05

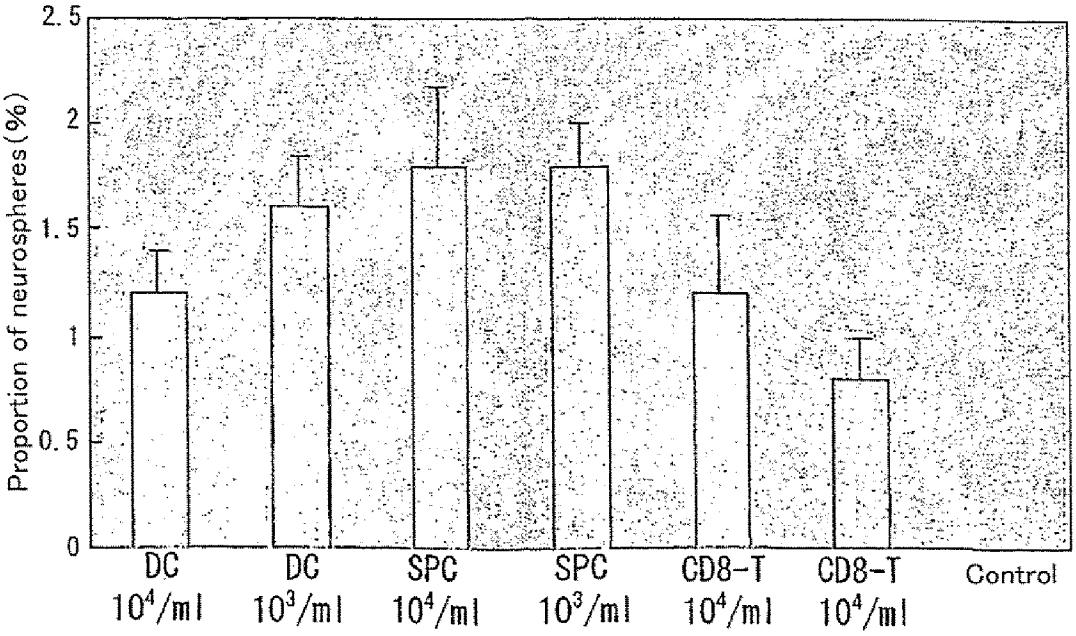
[Fig.6]


Proportion of neurospheres (%)
6
5
4
3
2
1
0
DC 10^5/ml
DC 10^4/ml
DC 10^3/ml
Control
NT-3 10ng/ml
NT-3 1ng/ml

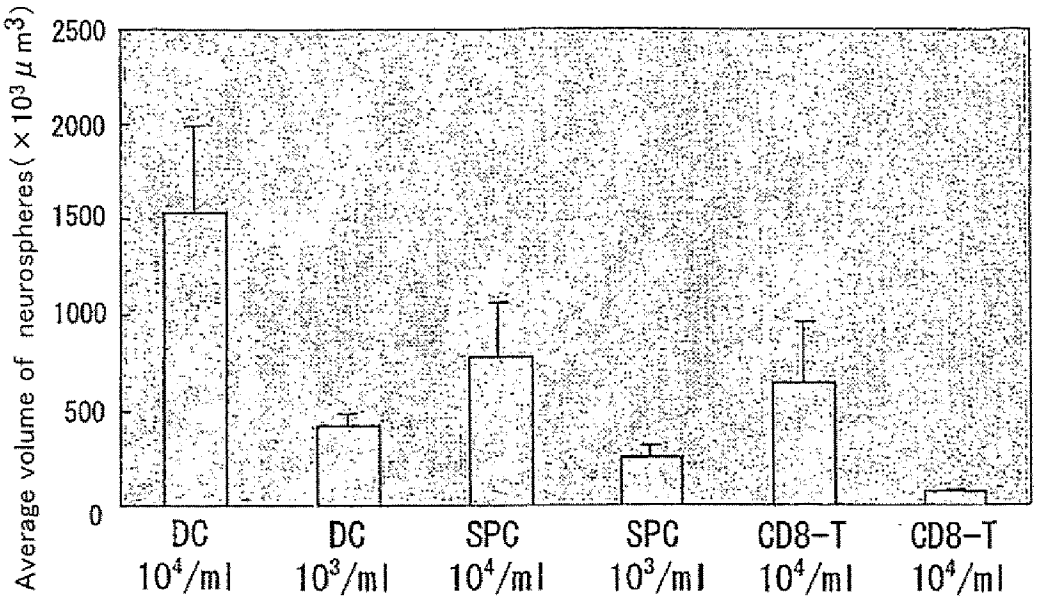
[Fig.7]


Average volume of neurospheres (× 10^3 μm^3)
3000
2500
2000
1500
1000
500
0
DC 10^5/ml
DC 10^4/ml
DC 10^3/ml
Control
NT-3 10ng/ml
NT-3 1ng/ml

[Fig.8]


Proportion of neurospheres (%)
2.5
2
1.5
1
0.5
0
DC 10⁴/ml
DC 10³/ml
SPC 10⁴/ml
SPC 10³/ml
CD8-T 10⁴/ml
CD8-T 10⁴/ml
Control

[Fig.9]


Average volume of neurospheres (×10³ μm³)
2500
2000
1500
1000
500
0
DC 10⁴/ml
DC 10³/ml
SPC 10⁴/ml
SPC 10³/ml
CD8-T 10⁴/ml
CD8-T 10⁴/ml

[NAME OF THE DOCUMENT] Abstract

[ABSTRACT]

[PROBLEMS TO BE SOLVED] To provide methods for efficiently inducing proliferation of neural stem cells *in vivo/in vitro*, which are the most important for transplantation therapy etc. of nerve injuries or nerve function insufficiency.

[METHODS TO SOLVE THE PROBLEMS] Mammalian nervous tissues containing neural stem cells are isolated, the neural stem cells are cultured in a culture medium containing growth factors, such as EGF and FGF, and then the neural stem cells are co-cultured with dendritic cells, such as an immature dendritic cell subset having the CD11c surface marker on the cell surface and blood cells, such as spleen cells and CD8-positive T cells. Or the neural stem cells after culture are cultured in the presence of GM-CSF. Alternatively, the neural stem cells after culture are cultured in the culture supernatant of dendritic cells and/or blood cells.